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STUDIES ON LIPID METABOLISM AND INCIDENCE OF AORTIC RUPTURE IN CHICKENS

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on Lipid Metabolism and Incidence of Aortic Rupture in Chickens" submitted by Tian-fuh Shen, B.S., in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

An investigation was undertaken to study the effects of dietary and hormonal factors on blood lipid constituents, fatty acid composition of serum lipids, hemoglobin content of blood, vascular lipidosis and occurrence of aortic rupture in chicks. Two experiments were conducted to determine the effects of the additions of cholesterol and cholic acid, and of dianisylhexene to rations containing suboptimal and optimal levels of magnesium or copper.

Hyperlipemia and hypercholesterolemia were noted when either cholesterol (1%) and cholic acid (0.2%) or dianisylhexene (0.02%) was added to the rations fed. Dianisylhexene administration was more effective in increasing serum lipid levels than cholesterol and cholic acid. A combination of dianisylhexene and cholesterol-cholic acid resulted in extremely high serum lipid concentrations.

Distribution of fatty acids in the serum lipids was affected by the inclusion of cholesterol and cholic acid or dianisylhexene in the rations fed. The addition of cholesterol and cholic acid resulted in a highly significant increase ($P < 0.01$) in the proportion of oleic acid with a corresponding decrease in the proportions of linoleic, stearic and palmitic acids. The administration of dianisylhexene resulted in an increase in the proportions of oleic and palmitic acids and a decrease in linoleic and stearic acids.

Feeding rations containing a suboptimal level of magnesium or copper had no appreciable influence on levels of total and free cholesterol, and total lipids in the serum or on the distribution of fatty acids in serum lipids.

Examination of the aorta of 6 week-old chicks showed that those fed rations supplemented with cholesterol-cholic acid, or dianisylhexene, alone or in combination, exhibited a high degree of lipidosis. The most severe lipidosis was noted in the groups receiving a combination of cholesterol-cholic acid and dianisylhexene.

Aortic rupture accounted for 4% of mortality in a group fed a complete ration supplemented with dianisylhexene and 11% of mortality in a group fed a copper-deficient ration supplemented with dianisylhexene. This indicated that a combination of copper deficiency and dianisylhexene affected the integrity of vascular system in chicks to a greater extent than other treatments used.

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INTRODUCTION

For many years nutritionists have maintained an interest in dietary and hormonal factors capable of causing hyperlipemia in animals. This interest has been stimulated by a relationship that seems to exist between high blood lipid levels and the occurrence of atherosclerosis.

The use of domestic fowl as a laboratory animal for studies on lipid metabolism has developed for a number of reasons. Gross arterial lesions similar to atherosclerosis in man occur spontaneously in poultry and can be induced by prolonged feeding of cholesterol or by administration of estrogenic hormones. In addition, chickens and turkeys are subject to aortic rupture and in some cases this condition may be induced experimentally by applying the same treatments as those used to produce hyperlipemia or hypercholesterolemia in animals.

Since many factors have been shown to be involved in the development of hyperlipemia and associated disorders, the present research was initiated to try to develop experimental treatments capable of inducing extremely severe hyperlipemia and hypercholesterolemia in chicks in the hope that this might, in turn, result in the development of atherosclerosis and aortic rupture.

REVIEW OF LITERATURE

Historical background

Cholesterol was identified as a major constituent of the atheromatous plaque by Vogel more than a century ago (Kritchevsky, 1967), but it was not until after the turn of the century that Anitschkow (1913) demonstrated that feeding cholesterol to rabbits resulted in establishment of atheromatous deposits in the aortas. This finding suggested that cholesterol was an important biochemical factor in the development of atherosclerosis. The effects of cholesterol on the production of hyperlipemia and cardiovascular lesions in chickens have been studied (Rodbard, Bolene, and Katz, 1951; Pick et al. 1952a; Pick, Stamler, and Katz, 1957; Stamler and Katz, 1950). The results obtained showed, in general, that the inclusion of cholesterol in the ration fed resulted in hyperlipemia, hypercholesterolemia, and an increased plasma ratio of cholesterol to phospholipid as compared with chickens not given cholesterol.

Early studies on the influence of estrogen on lipid metabolism in chickens have been reported by Lorenz, Chaikoff and Entenman (1938), Entenman, Lorenz, and Chaikoff (1940), and Lindsay et al. (1946), who noted that administration of estrogen caused an increase in neutral fat, phospholipid and cholesterol content of the blood. The relationship between estrogen administration and the occurrence of atherosclerosis was not recognized until Lindsay et al. (1946) observed that atherosclerosis occurred in chicken having a prolonged hyperlipemia induced by implantation of estrogens. With these discoveries, and the use of more refined analytical procedures, numerous studies of the

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effect of cholesterol and estrogen administration on lipid metabolism and its relationship to the induction of atherosclerosis have been conducted during the last twenty years.

In addition to agents causing hyperlipemia and hypercholesterolemia, other factors may be involved in the production of atherosclerosis. Vitale et al. (1957) and Hellerstein et al. (1957) reported that magnesium might be involved in lipid metabolism and the production of atherosclerosis. O'Dell et al. (1961) noted that a copper deficiency resulted in aortic rupture in chicken.

It is apparent that many factors may be involved in the occurrence of hyperlipemia and associated disorders. As indicated above, inclusion of cholesterol and cholic acid in the diet, administration of estrogens, and deficiencies of magnesium and copper have been implicated. It is proposed to review some of the experiments in which a relationship between these factors and blood lipid levels, fatty acid metabolism and changes in the vascular system have been established.

Effects of cholesterol, cholic acid and estrogen on levels of blood lipids of poultry

Inclusion of cholesterol in the ration fed causes an increase in the lipid and cholesterol levels of the blood. Stamler and Katz (1950) noted that the inclusion of 2% of cholesterol in a mash diet supplemented with 5% of cottonseed oil resulted in 4 to 10-fold increases in total plasma cholesterol with 3 to 5-fold increases in the ratio of total cholesterol to phospholipid. Rodbard et al. (1951) observed that the tendency toward the development of hypercholestero-

lemia and atherosclerosis was correlated with the amount of cholesterol ingested per kilogram of body weight of chicks. Leveille, Tillotson, and Sauberlich (1963) noted that inclusion of 0.3% of cholesterol in a low protein (12%) diet or 2% of cholesterol in a high protein (25%) diet resulted in an increase in plasma and liver cholesterol and glyceride levels, but a decrease in α-lipoprotein cholesterol and phospholipid levels. Blomstrand and Christensen (1963) also observed that the cholesterol content of plasma and liver increased with the addition of cholesterol to the ration. Similar results were obtained in studies with rabbits and rats (Page and Bernhard, 1935; Hegsted et al., 1957).

The extent of the effect of dietary cholesterol on plasma cholesterol levels depends, at least in part, on the composition and level of fat used in the ration. Hegsted, Gotsis, and Stare (1960) observed that fats containing high levels of saturated fatty acids promoted hypercholesterolemia. This effect was counteracted to some extent by inclusion of unsaturated fatty acids in the ration in place of saturated fatty acids. Beeler, Rogler, and Quackenbush (1962) noted that adding cholesterol to a diet containing a low level of fat increased serum cholesterol and total lipids to a lesser extent than when added to a diet containing a high level of fat. Blomstrand and Christensen (1963) showed that cockerels fed a ration containing 1% of cholesterol supplemented with 3% of peanut oil had higher cholesterol levels in the plasma than those given 1% of cholesterol without oil.

Increases in plasma lipid levels resulting from cholesterol feeding may be enhanced by the addition of cholic acid to the ration. Member, Bruger, and Oppenheim (1944) noted that the cholesterol

content of the blood and aortas of rabbits fed diets containing cholesterol and cholic acid was markedly increased as compared to those fed diets containing cholesterol alone. Page and Brown (1952) indicated that feeding cholesterol and cholic acid to normal rats elicited hypercholesterolemia with 2.5 to 5-fold increases in total serum cholesterol. Hegsted et al. (1957, 1960) confirmed these findings in studies with rats and chicks and noted a significant interaction between various levels of cholesterol and cholic acid. It was suggested that the interaction might be due to the effect of cholic acid on cholesterol absorption or on cholesterol metabolism.

The effects of estrogen administration on blood lipid levels of the chicken have been extensively studied. Early investigations on the influence of estrogen on lipid metabolism showed that estrogen implantation resulted in hyperlipemia characterized by elevation of the concentration of neutral fats, phospholipid, and cholesterol in the blood (Lorenz et al.,1938; Entenman et al.,1940; Lindsay et al., 1946). Among the plasma lipid fractions, it was observed that the greatest increase occurred in the neutral fat fraction. Diethylstilbestrol gave the greatest response of any of the estrogenic substances tested; the estrogens tested were estrone, estradiol, estradiol benzoate, ethinyl estradiol, and diethylstilbestrol. Pick et al. (1952a,b; 1957) noted that estradiol benzoate administration resulted in a pronounced increase in total cholesterol levels of the blood and a marked increase in phospholipid levels. Tennent et al. (1959) reported that implantation of diethylstilbestrol in cockerels caused a significant rise in plasma cholesterol levels and a greater increase in plasma phospholipid concentrations. More recently, it

was observed that a pronounced hyperlipemia was produced in chickens treated with diethylstilbestrol (Blomstrand and Christensen, 1963). Heald and Rookledge (1964) also noted that estrogen treatment increased the level of free fatty acids in plasma simultaneously with those of total lipids and lipoprotein in the immature fowl.

The influence of cholesterol feeding and estrogen administration on serum lipid levels appears to be additive. When cholesterol and estrogen were administered concomitantly, total serum cholesterol levels of chickens were elevated as compared with those given either treatment alone (Pick et al., 1952a; 1952b; 1957). Moreover, it was noted that the plasma ratio of cholesterol to phospholipid was at or near normal levels when birds were fed estrogen and cholesterol simultaneously, whereas chicks fed cholesterol without estrogen showed uniformly elevated ratios.

Effects of cholesterol and estrogen on fatty acid metabolism

Inclusion of cholesterol in a diet results in a change in fatty acid distribution of the lipids with the most pronounced change occurring in the proportion of oleic acid present. Luddy, Barford, and Riemenschneider (1958) noted that cholesterol esters, glycerides and phospholipids of human atheroma contained greater amounts of oleic acid and less dienoic and tetraenoic acids than did comparable fractions from normal human sera. Blomstrand and Christensen (1961) observed a significant increase in the percentage of oleic acid and a corresponding decrease in stearic acid present in the cholesterol ester and triglyceride fractions of aortic lipids of cockerels fed a ration containing cholesterol. No apparent changes in the proportions

of linoleic acid were noted in either fraction, and the fatty acid composition of the phospholipid fraction of the aortic lipids was similar to the controls. However, Fisher and Feigenbaum (1960) reported that dienoic and tetraenoic acids concentration of the lipids from portions of chicken aorta containing atheromatous plaques was lower than that from clear areas of the aorta. Leveille et al. (1963) noted that supplementing the diet of growing chicks with cholesterol resulted in a significant elevation in oleic acid and a decline in arachidonic acid levels of the plasma and liver lipids. They also noted that, in general, cholesterol feeding tended to decrease the linoleic acid and palmitic acid content of plasma and liver lipids. Blomstrand and Christensen (1963) and Chung, Ning, and Tsao (1966) confirmed these findings and provided further evidence that a rise in oleic acid percentage in cholesterol-fed birds was associated with a decrease in the percentage of stearic acid present.

Alteration in the fatty acid composition of plasma or liver lipids of animals treated with diethylstilbestrol appeared to be similar to those fed diets containing cholesterol. Blomstrand and Christensen (1963) observed that plasma or liver lipids from cockerels implanted with diethylstilbestrol showed a marked increase in oleic acid and a decrease in stearic acid as compared with non-treated cockerels. Chung et al. (1966) noted that, in addition to changes in oleic acid and stearic acid levels, estrogen administration resulted in an increase in palmitic acid and a decrease in linoleic acid levels. Similar changes in fatty acid composition were observed in studies with turkeys (Chung, Munday, and Lien, 1967).

Although the influence of cholesterol and estrogen administra-

tion on hyperlipemia appears to be additive, as described previously, no additive effect occurs with respect to alteration of the distribution of fatty acids. Chung et al. (1966) observed that the changes in fatty acid composition of plasma lipids of cockerels treated with diethylstilbestrol were similar to those treated with cholesterol and diethylstilbestrol.

Effects of estrogen on blood hemoglobin levels and defects in the vascular system of poultry

Estrogen administration appears to affect the hemoglobin content of the blood. Harmon (1936) observed a decline in blood hemoglobin concentration of laying hens as compared to non-laying hens and suggested that blood levels of estrogen played an important role in the effect. Ramsay and Campbell (1956) noted that the injection of estradiol benzoate into pullets resulted in a decrease in hemoglobin content of the blood.

The administration of estrogen also has an effect on the incidence of aortic rupture in avian species. Pritchard, Henderson, and Beall (1958) noted that male turkeys died of dissecting aneurysms in a commercial flock that had been implanted with diethylstilbestrol pellets. Beall et al. (1963) were able to induce aortic rupture by implanting turkeys with diethylstilbestrol at weekly intervals and noted that the incidence increased when the protein content of the ration was low. Krista, Waibel, and Burger (1961; 1965) fed turkeys a high fat, low protein diet and found that the injection of diethylstilbestrol increased the occurrence of aortic rupture. Simpson and Harms (1966) confirmed this observation using diethylstilbestrol

administered orally.

The influence of estrogen on the occurrence of aortic rupture in chickens appears to be inconsistent. Carlton and Henderson (1965) showed that diethylstilbestrol did not significantly affect the incidence of arterial rupture when added to a copper-deficient diet. However, Pick, Stamler, and Katz (1958) showed that estrogen, when added to the high energy, low protein ration supplemented with cholesterol, caused ulceration and hemorrhage in the aorta of cockerels fed the ration from 9 to 14 weeks of age. Two of the treated cockerels died of dissecting aneurysms.

Effects of dietary magnesium on lipid metabolism

Magnesium is involved in a wide variety of physiological and biochemical functions, but its role in lipid metabolism is obscure. Vitale et al. (1957) and Hellerstein et al. (1957, 1960) noted that when rats were fed a high-fat diet with an adequate level of magnesium and containing cholesterol and cholic acid, a syndrome typical of a magnesium deficiency was produced. Increasing the level of magnesium in the ration was effective in preventing the development of the deficiency symptoms. In addition, it was observed that the higher levels of magnesium in the ration partially prevented the lipid deposition in the heart valve and aorta that was noted when lower levels of magnesium were fed.

Although dietary magnesium has been shown to be related to lipid deposition in the cardiovascular system, Vitale et al. (1959) and Hellerstein et al. (1960) reported that levels of serum cholesterol in rats were not apparently affected by the intake of magnesium.

However, serum magnesium levels were significantly lowered in rats made hypercholesterolemic by feeding cholesterol and cholic acid.

In studies with rabbits, it was observed that the effect of magnesium on lipid metabolism was inconsistent. Neal and Neal (1962) fed rabbits a high-fat diet supplemented with cholesterol, and noted that the aortic atheroma and serum turbidity caused by hyperlipemia could be reduced by the administration of magnesium sulfate to the drinking water. On the contrary, Adams et al. (1964) reported that magnesium sulfate or magnesium lactate administration orally had no protective effect against the development of hypercholesterolemia, or aortic atheroma in rabbits fed cholesterol.

No information is available on relationship existing between dietary magnesium and cholesterol levels in the blood of chickens.

Effects of dietary copper on the vascular system of poultry

Dietary copper has been shown to be involved in the maintenance of a normal vascular system in animals. O'Dell et al. (1961) showed that copper was essential for the maintenance of the connective tissue of vascular vessels of chickens. They reported that a high level of mortality, without apparent outward symptoms, occurred in chickens fed a copper-deficient ration. Upon autopsy, internal hemorrhages associated with rupture of the main blood vessels were observed. Histological studies showed a derangement of connective tissue metabolism with the major defect in elastin tissue. Accumulation of an unidentified material in the interlaminar spaces in copper-deficient aortas occurred. It was suggested that "the role of copper in connective tissue metabolism is more important for survival than is

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its role in hemopoiesis". The importance of copper in maintaining a normal vascular system in poultry has been confirmed (Carlton and Henderson,1963, 1965; Simpson and Harms, 1964).

The occurrence of aortic rupture as a result of a deficiency of copper is not confined to poultry. In studies with swine, internal hemorrhages due to rupture of the aorta, coronary or pulmonary arteries were observed when a copper-deficient diet was fed (Shields et al., 1962). It was concluded that copper is required for the maintenance of the structural elements of the cardiovascular vessels of swine.

In view of the importance of copper in maintaining normal vascular development in animals, attempts have been made to ascertain the cause of rupture of blood vessels in different animals. Starcher, Hill, and Matrone (1964) noted that the elastin content of the aorta of chicks fed copper-deficient rations was lower than that of chicks fed rations containing adequate amounts of copper. It was suggested that the deficiency had an effect on elastin biosynthesis. O'Dell et al. (1966) observed that the concentration of desmosine, a crosslinking agent of elastin, was decreased in copper deficient aorta, thus providing further evidence of the importance of copper in relation to connective tissue metabolism.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

Two experiments were conducted to study the effects of dietary and hormonal factors on blood lipid levels, fatty acid composition of serum lipids, hemoglobin content of blood, lipidosis of the aorta, and incidence of aortic rupture in chicks. In the first experiment, dietary variables included the additions of cholesterol and cholic acid, and of dianisylhexene to rations containing suboptimal or optimal levels of magnesium. In the second experiment, the dietary variables, cholesterol, cholic acid and dianisylhexene, were imposed on diets deficient or adequate in copper content and containing adequate levels of magnesium.

Status of the problem

Losses in poultry flocks from a condition known as aortic rupture have been noted quite commonly. In many instances, when such mortality occurs, no explanation can be offered for its occurrence. Since rupture of the aorta implies a loss in the integrity of the vascular system, it is possible that the condition may in some way be related to other disorders of the vascular system such as atherosclerosis or hyperlipemia.

It is recognized that atherosclerosis is partly the result of lipid accumulation in the intimal layer of the aorta. The deposition of lipid in the aorta may be due to some fault in lipid metabolism resulting from elevated blood lipid levels or from changes in the composition of the lipids.

A large number of factors are capable of causing hyperlipemia in animals. They include fat and protein levels of the ration,

addition of cholesterol and cholic acid to the ration and the administration of estrogenic hormones. In the chicken, these factors have been shown to result in hyperlipemia, hypercholesterolemia and changes in the fatty acid composition of blood lipids. An increased incidence of atherosclerosis has also been noted.

There is some evidence that levels of magnesium in the ration may also affect lipid metabolism and that copper deficiency affects the integrity of vascular system. Thus, it was thought that dietary magnesium and copper might be involved along with cholesterol, cholic acid and estrogenic hormones in the development of hyperlipemia and associated disorders of the vascular system of poultry.

The present experiments were undertaken to try to develop dietary treatments that would result in rapid appearance of severe hyperlipemia, atherosclerosis and aortic rupture in young chickens. The variables employed included the feeding of high levels of cholesterol and cholic acid and inclusion of dianisylhexene, an estrogenic hormone, in purified rations containing either suboptimal or optimal levels of magnesium or copper. Dianisylhexene was selected as the estrogenic hormone because it had been observed previously that this compound caused extremely severe hyperlipemia in laying hens and resulted in a high level of mortality. The interrelationships existing among these factors in altering serum lipid constituents and their effects on lipidosis of the aorta and the occurrence of aortic rupture were investigated.

Experimental (General)

Experimental design

Day-old Dominant White x White Plymouth Rock chicks, hatched at the University of Alberta Poultry Research Farm, were randomly assigned to the experimental groups. The chicks were maintained in electrically heated, thermostatically controlled, battery brooders with raised screen floors throughout a six week experimental period.

The composition of the basal ingredients used in the experimental rations is shown in Table 1. The basal constituted 98% of each of the experimental rations and the remaining 2% was made up of sucrose and other supplements added to the rations. Inclusion of dietary variables was made at the expense of the 2% of sucrose in the ration. When included in the rations, cholesterol was added at a level of 1%, cholic acid at 0.2% and dianisylhexene at 0.02%. The mineral mix used was similar to that used by Renner (1964) except that all of the copper and one half of the magnesium were omitted. Feed and water were supplied *ad libitum*.

The chicks were wing-banded and weighed individually at weekly intervals. Mortality and incidence of aortic rupture were recorded.

Blood samples from 5 chicks per group were collected at 2, 4 and 6 weeks of age for determination of the levels of total and free cholesterol and total lipids in the serum. The samples were taken from the jugular vein. Chicks were fasted 12 hours before taking blood samples to reduce post-absorptive effects on blood constituents.

Table 1. Composition of basal ingredients

Ingredients	%
Sucrose	54.35
Stabilized tallow	10.0
Casein (vitamin-free)	18.0
Gelatin	10.0
Vitamin mix[1]	0.35
Choline chloride	0.15
DL-methionine	0.10
Mineral mix[2]	1.25
Calcium carbonate	1.50
Dicalcium phosphate	1.70
Sodium chloride	0.60
Total	98.00

[1]Vitamins were added at the following levels per kilogram of ration: vitamin A, 4062 IU; vitamin D_3, 496 ICU; vitamin E, 20 IU; vitamin K (menadione), 2 mg; thiamine, 3 mg; riboflavin, 6 mg; calcium pantothenate, 20 mg; niacin, 50 mg; pyridoxine, 6 mg; biotin, 0.2 mg; folic acid, 2.4 mg; vitamin B_{12}, 0.02 mg.

[2]Minerals were added at the following levels per kilogram of ration: KH_2PO_4, 9.3 g; $MgSO_4.7H_2O$, 2.47 g; KI, 2.9 mg; $FeSO_4.7H_2O$, 0.28 g; $ZnCl_2$, 125 mg; $CoCl_2.6H_2O$, 1.7 mg; $Na_2MoO_4.2H_2O$, 8.3 mg; Na_2SeO_3, 0.22 mg; $MnSO_4.H_2O$, 0.37 g.

Analytical procedures

Individual blood samples were collected in a test tube without anticoagulant and allowed to clot at room temperature. The samples were chilled in a refrigerator at 2^{o}C, and were then centrifuged in a refrigerated centrifuge at 2100 rpm for 20 minutes. The supernatant serum was removed and stored at 2^{o}C until the analyses were completed.

Cholesterol was extracted from serum with acetone-ethanol (1:1). Determination of total and free cholesterol in the serum was conducted by the colorimetric method developed by Searcy and Bergquist (1960).

Total serum lipids were extracted with chloroform-methanol (2:1), and determined quantitatively by the procedures described by Sperry and Brand (1955). The extracted lipid, after total lipids were determined, was placed in a 4-ml vial, sealed under nitrogen, and stored at -18^{o}C for subsequent determination of fatty acid composition.

Fatty acid analyses were carried out by gas-liquid chromatography. Lipids extracted from serum were methylated with boron fluoride-methanol according to the method of Morrison and Smith (1964). The fatty acid methyl esters were then analyzed in an Aerograph model A_{90}-P_3 gas chromatograph with thermal conductivity detector. The aluminum column, 10' x 0.25", was packed with 20% diethylene glycol succinate on 60-80 mesh firebrick and operated at 210^{o}C. The detector and injector were operated at 250^{o}C and 260^{o}C, respectively. Helium served as carrier gas; flow rate was maintained at 100 ml per minute. The appearance of each fatty acid was recorded with a Photovolt Microcord model 44 and the fatty acids were determined by an

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electronic integrator. The levels of individual fatty acids were corrected by the use of standards.

Hemoglobin content of the whole blood of 10 chicks from each group was determined by using the acid hematin method outlined by Cohen and Smith (1919).

The degree of lipidosis of the aorta was determined by visual appraisal after staining with Sudan IV, according to the method of Holman et al. (1958).

Statistical analysis

Analysis of variance was performed on the data by an IBM 360 model 67 computer using the library program CS017, University of Alberta (1968). Significant mean differences among treatment groups were assessed by using Duncan's new multiple-range test (Steel and Torrie, 1960).

EXPERIMENT I

Object

To study the effects of the addition of atherogenic substances to rations containing suboptimal or optimal levels of magnesium on total serum lipids, total and free cholesterol levels in the serum, fatty acid composition of serum lipids, blood hemoglobin content, vascular lipidosis, and aortic rupture in chicks.

Experimental

One hundred and eighty day-old Dominant White x White Plymouth Rock chicks were randomly assigned to six experimental groups of thirty birds each. The composition of the ration fed to each group is outlined in Table 2. The control ration (Group 1) contained adequate amounts of magnesium (488 mg/kg) and no supplementary cholesterol and cholic acid. Rations for Groups 2 and 3 were supplied with cholesterol and cholic acid and contained optimal (488 mg/kg) and suboptimal (244 mg/kg) levels of magnesium, respectively. Groups 4 and 5 were fed rations similar to Groups 2 and 3 except that 0.02% of dianisylhexene was added to the rations. The ration fed to Group 6 was of the same composition as that fed to Group 1 except that 5% of corn oil and 5% of sucrose were substituted for 10% of stabilized tallow. All rations were supplemented with sufficient copper to meet the requirements of the chick.

Results and Discussion

Changes in levels of total cholesterol, free cholesterol and total lipids of blood serum and in the hemoglobin content of the

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Table 2. Composition of experimental rations used in Experiment 1.

Ingredients	Treatment group 1	2	3	4	5	6
Basal ingredients	98	98	98	98	98	98
Sucrose	1.750	0.55	0.798	0.53	0.778	1.750
$CuSO_4.5H_2O$	0.002	0.002	0.002	0.002	0.002	0.002
$MgSO_4.7H_2O$	0.248	0.248	--	0.248	--	0.248
Cholesterol	--	1.0	1.0	1.0	1.0	--
Cholic acid	--	0.2	0.2	0.2	0.2	--
Dianisylhexene	--	--	--	0.02	0.02	--

whole blood resulted from the dietary variables employed (Table 3). Cholesterol-cholic acid supplementation (Groups 2 and 3) resulted in a significant increase ($P < 0.05$) in total and free cholesterol levels and a marked increase (statistically nonsignificant because of large variation within treatment) in total lipid levels of the serum as compared with chicks fed rations without cholesterol and cholic acid (Group 1). The addition of dianisylhexene to the rations containing cholesterol and cholic acid (Groups 4 and 5) further increased ($P < 0.01$) the levels of total and free cholesterol and total lipids of the serum and resulted in severe hyperlipemia and hypercholesterolemia regardless of dietary magnesium levels. The amount of increase attributable to dianisylhexene administration was approximately 2.5 times for total serum cholesterol, 4 times for free cholesterol and 6.5 times for total serum lipids (Groups 4 and 5 vs. Groups 2 and 3).

Table 3. Effects of treatments on serum lipid constituents and hemoglobin content of blood of chicks

Group number	Age of chick week	Serum lipid constituents: Total cholesterol mg/100 ml	Free cholesterol mg/100 ml	Total lipids mg/100 ml	TC/TL ratio[1]	FC/TC ratio[2]	Hemoglobin g/100 ml
1	2	188.4	23.0	643	0.29	0.12	9.56
	4	208.7	22.2	532	0.39	0.11	10.73
	6	208.5	20.1	652	0.32	0.10	10.84
	avg	201.8	21.7	609	0.33	0.11	10.37
2	2	413.6	44.8	962	0.43	0.11	9.32
	4	598.1	95.4	1796	0.33	0.16	10.84
	6	588.4	78.9	1339	0.44	0.13	10.71
	avg	533.3	73.0	1366	0.40	0.13	10.29
3	2	425.8	55.2	1152	0.37	0.13	9.28
	4	544.4	62.1	1252	0.43	0.11	10.31
	6	649.0	102.8	1782	0.36	0.16	12.38
	avg	539.7	73.4	1395	0.39	0.13	10.66
4	2	915.6	201.0	6036	0.15	0.22	7.38
	4	1285.8	233.4	7552	0.17	0.18	7.81
	6	1873.9	364.4	14129	0.13	0.19	8.18
	avg	1358.4	266.3	9239	0.15	0.20	7.79
5	2	973.0	158.7	7003	0.14	0.16	7.18
	4	1482.6	556.1	7575	0.20	0.37	7.13
	6	1831.8	362.9	11864	0.15	0.20	8.89
	avg	1429.1	359.2	8814	0.16	0.24	7.73
6	2	251.7	25.1	769	0.33	0.10	9.44
	4	234.4	18.8	844	0.28	0.08	10.11
	6	189.8	14.8	622	0.30	0.08	11.76
	avg	225.3	19.6	745	0.30	0.09	10.44

[1] Ratio of total cholesterol to total lipids.

[2] Ratio of free cholesterol to total cholesterol.

The ratios of free cholesterol to total cholesterol and of total cholesterol to total lipids were calculated. The ratio of free cholesterol to total cholesterol tended to increase when chicks were fed rations containing either cholesterol-cholic acid alone or in combination with dianisylhexene regardless of levels of magnesium in the ration. The addition of a combination of cholesterol, cholic acid and dianisylhexene to the rations resulted in the highest ratio of free cholesterol to total cholesterol in the serum. The generally low ratios of free cholesterol observed in the experiment indicated that most of the cholesterol in the serum was in the esterified form.

The ratio of total cholesterol to total lipids was increased slightly when cholesterol and cholic acid were added to the rations fed, but the addition of dianisylhexene resulted in a marked decrease in the ratio. This suggested that cholesterol-cholic acid feeding caused an increase in the cholesterol fraction of the serum, whereas dianisylhexene resulted in an increase in other lipid fractions, possibly in the triglyceride moiety.

Type of dietary fat added to the ration apparently did not influence total and free cholesterol levels or total lipid levels of the serum in the absence of dietary cholesterol and cholic acid. It was observed that levels of total and free cholesterol, and of total lipids in the serum of those chicks fed corn oil (Group 6) were similar to those fed stabilized tallow (Group 1). No apparent differences in ratios of free cholesterol to total cholesterol and of total cholesterol to total lipids were noted.

No appreciable differences were observed in total and free

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cholesterol and total lipid levels of the serum when chicks were fed rations which varied in magnesium content; 488 and 244 mg/kg, respectively. Feeding rations containing a suboptimal level of magnesium had no effect on the severity of hyperlipemia and hypercholesterolemia noted. This observation confirms the reports of Vitale et al. (1959) and Hellerstein et al. (1960) who reported, in studies with rats, that serum cholesterol levels were not apparently affected by magnesium intake.

Despite the severity of hyperlipemia and hypercholesterolemia observed in the groups fed cholesterol, cholic acid and dianisylhexene, no atherosclerotic plaques were noted in the aortas of the experimental chicks. There was, however, some evidence that lipidosis of the vascular system occurred when these supplements were included in the ration. When sections of the aorta and brachiocephalic artery were examined for lipid deposition by staining with Sudan IV, it was found that a high concentration of lipids was deposited in the intima as shown by the deep red color of the stained sections (Figure 1). Levels of magnesium in the rations fed apparently had no effect on lipid deposition in the aortic intima. Vitale et al. (1957) and Hellerstein et al. (1957, 1960) observed that high dietary magnesium (4 to 8 times of the requirement) caused a reduction in lipid deposition in the aorta and heart valve in rats made hypercholesterolemic by supplementing the diet with cholesterol and cholic acid. The lack of effect in the present experiment may be related to the levels of magnesium used. Concentrations well in excess of the requirements may be necessary for a reduction in lipid deposition to occur.

Figure 1. The effect of treatments on lipidosis of the aorta (upper row) and brachiocephalic arteries of 6 week-old chicks (Experiment I).

The effect of treatment on the distribution of fatty acids in serum lipids is presented in Table 4. The addition of cholesterol and cholic acid to the rations fed resulted in a highly significant increase ($P < 0.01$) in the proportion of oleic acid (18:1) when compared with chicks given rations without cholesterol and cholic acid. The increase in oleic acid levels was accompanied by a decrease ($P < 0.05$) in the proportions of palmitic (16:0), stearic (18:0) and linoleic (18:2) acids.

Changes in fatty acid composition induced by a combination of cholesterol, cholic acid and dianisylhexene seemed to follow a similar pattern to that noted when cholesterol and cholic acid were fed with the exception of palmitic and linoleic acids. The addition of dianisylhexene caused a significant increase ($P < 0.05$) in palmitic acid and a decrease ($P < 0.05$) in linoleic acid as compared with chicks fed a ration supplemented with cholesterol and cholic acid alone. Level of magnesium in the rations fed had no effect on the distribution of fatty acids in serum lipids.

Fatty acid composition of serum lipids was significantly influenced by the pattern of fatty acids in the dietary fat (Group 1 vs. Group 6). The distribution of fatty acids in the serum tended to reflect the composition of fatty acids in the diet. Serum lipids from chicks fed the ration containing corn oil showed a marked increase in the proportion of linoleic acid and a decrease in oleic acid.

Level of hemoglobin in the blood was not affected by the addition of cholesterol and cholic acid to the ration fed (Table 3). The addition of dianisylhexene, however, caused a significant decrease

Table 4. Effect of treatments on the distribution of fatty acids in serum lipids

Group number	Age of chick week	Fatty acids[1] 14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0
		%							
1	2	0.93	25.03	5.38	15.94	46.48	4.79	1.06	0.36
	4	0.67	22.78	4.46	15.96	44.71	10.80	0.23	0.37
	6	0.42	20.77	4.37	16.14	48.92	9.16	0.15	0.17
	avg	0.67	22.86	4.74	16.01	46.70	8.25	0.48	0.30
2	2	0.70	19.79	5.47	13.26	51.67	7.89	0.92	0.28
	4	0.44	13.68	4.57	9.86	64.81	5.48	0.49	0.65
	6	0.38	13.18	4.28	13.74	61.95	5.77	0.25	0.43
	avg	0.51	15.55	4.77	12.29	59.48	6.38	0.55	0.45
3	2	0.53	17.31	5.86	12.27	55.22	8.33	0.18	0.26
	4	0.51	15.17	4.12	11.75	60.06	7.46	0.49	0.42
	6	0.48	13.49	4.16	9.97	64.23	6.19	0.69	0.77
	avg	0.51	15.32	4.71	11.33	59.84	7.33	0.45	0.48
4	2	0.49	24.38	6.90	8.96	54.41	3.79	0.52	0.51
	4	0.43	20.22	4.65	11.94	57.55	4.01	0.48	0.69
	6	0.67	22.74	5.04	9.58	58.25	2.55	0.58	0.56
	avg	0.53	22.45	5.53	10.16	56.74	3.45	0.53	0.59
5	2	0.53	25.83	6.72	7.54	54.52	4.24	0.30	0.31
	4	0.47	21.46	5.52	10.07	57.20	3.70	0.77	0.79
	6	0.55	21.74	4.82	8.16	60.96	2.75	0.51	0.49
	avg	0.52	23.01	5.69	8.59	57.56	3.56	0.53	0.53
6	2	1.04	25.74	2.73	20.93	25.87	23.13	0.54	tr[2]
	4	0.60	24.52	1.49	22.95	21.52	28.40	0.24	0.27
	6	0.28	23.62	1.60	22.86	20.24	31.38	tr	tr
	avg	0.64	24.63	1.94	22.25	22.54	27.64		

[1]Carbon chain length:number of double bonds.
[2]tr: trace ($<$ 0.1%).

($P < 0.05$) in the hemoglobin content. This may have been related to the extremely high levels of blood lipids in the groups receiving dianisylhexene. Type of dietary fat had no appreciable influence on blood hemoglobin content (Group 1 vs. Group 6).

Body weight gain was not affected by feeding of cholesterol and cholic acid (Table 5). Growth, however, was depressed when dianisylhexene was added to the rations fed. Rate of growth of chicks fed suboptimal levels of magnesium was lower than that of chicks fed adequate levels of magnesium regardless of whether cholesterol-cholic acid or dianisylhexene was added to the rations. The depression in rate of growth was accentuated when dianisylhexene was added to a ration of suboptimal magnesium content (Group 5).

All chicks that died during the course of the experiment were examined for evidence of aortic rupture or internal hemorrhage. No cases of aortic rupture were noted in this experiment.

Table 5. Effects of treatments on growth rate, mortality, and occurrence of aortic rupture

Group number	Body weight 6 weeks g	Mortality %	Occurrence of aortic rupture %
1	552	7	0
2	547	0	0
3	495	13	0
4	450	7	0
5	377	33	0
6	499	16	0

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Summary

1) Feeding of cholesterol (1%) and cholic acid (0.2%) resulted in a significant increase ($P < 0.05$) in total and free cholesterol and a pronounced, but statistically non-significant, increase in total lipid levels of the serum of chickens. A highly significant increase ($P < 0.01$) in the proportion of oleic acid (18:1) with corresponding decreases in linoleic (18:2), stearic (18:0) and palmitic (16:0) acids was observed in chicks fed rations supplemented with cholesterol and cholic acid.

2) The addition of dianisylhexene to the ration containing cholesterol and cholic acid further increased ($P < 0.01$) the levels of total and free cholesterol and total lipids in the serum. Little change in the distribution of fatty acids was noted except for a decrease in the proportion of linoleic acid and an increase in palmitic acid.

3) The inclusion of cholesterol and cholic acid alone or in combination with dianisylhexene in the rations fed resulted in lipidosis of the intima of the aorta.

4) Levels of dietary magnesium had no influence on total and free cholesterol and total lipid levels of the serum or on the distribution of fatty acids in the serum.

5) The addition of dianisylhexene to the ration caused a depression in the hemoglobin content of the whole blood.

6) Depressed growth rate and high mortality were observed in the group fed a suboptimal magnesium ration supplemented with cholesterol-cholic acid and dianisylhexene.

7) No occurrence of aortic rupture was observed in the experiment.

EXPERIMENT II

Object

The results of the previous experiment indicated that the addition of cholesterol, cholic acid and dianisylhexene to a purified ration resulted in severe hyperlipemia and hypercholesterolemia but was not effective in causing aortic rupture to occur in chicks. Since it had been reported (O'Dell et al. 1961) that copper was essential for the maintenance of a normal vascular system in chicks, an experiment was undertaken to study the effects of the addition of the same atherogenic substances to rations deficient or adequate in copper content.

Experimental

Two hundred and sixteen day-old chicks were randomly divided into eight groups of 27 birds each. The basal ingredients for the experiment were the same as those used in Experiment 1. The rations used are shown in Table 6. Magnesium sulfate was added to all rations to supply an adequate level of magnesium (488 mg/kg). When used, cupric sulfate ($CuSO_4.5H_2O$) was added at a level to supply 5 ppm of copper in the rations. This is a level slightly in excess of the requirements of the chick. In order to avoid the addition of trace amounts of copper in the water supply, the drinking water was demineralized by passing distilled water through a demineralizer and was supplied to the chicks in stainless steel troughs.

Results and Discussion

The effects of treatments on levels of total and free

Table 6. Composition of experimental rations used in Experiment 2.

Ingredients	Treatment group 1	2	3	4	5	6	7	8
Basal ingredients	98	98	98	98	98	98	98	98
Sucrose	1.75	0.55	1.752	0.552	1.730	0.53	1.732	0.532
$MgSO_4 \cdot 7H_2O$	0.248	0.248	0.248	0.248	0.248	0.248	0.248	0.248
Cholesterol	--	1.0	--	1.0	--	1.0	--	1.0
Cholic acid	--	0.2	--	0.2	--	0.2	--	0.2
Dianisylhexene	--	--	--	--	0.02	0.02	0.02	0.02
$CuSO_4 \cdot 5H_2O$	0.002	0.002	--	--	0.002	0.002	--	--

cholesterol of serum, total lipids of serum, and hemoglobin content of whole blood are presented in Table 7. As in Experiment 1, the addition of cholesterol and cholic acid to the rations fed (Groups 2 and 4) resulted in a significant increase ($P < 0.05$) in the levels of total and free cholesterol in the serum, and total serum lipid levels were markedly increased (statistically non-significant) as compared with chicks fed rations without supplementary cholesterol and cholic acid (Groups 1 and 3). The addition of dianisylhexene alone to rations adequate or deficient in copper content (Groups 5 and 7) resulted in a highly significant increase ($P < 0.01$) in total and free cholesterol as well as total lipid levels of the serum as compared with the groups fed cholesterol and cholic acid (Groups 2 and 4). A combination of cholesterol-cholic acid and dianisylhexene (Groups 6 and 8) resulted in a further increase in the concentrations of total and free cholesterol and total lipids in the serum as compared with chicks fed rations

supplemented with either cholesterol-cholic acid or dianisylhexene alone regardless of the levels of copper in rations. Thus, the effects of a combination of cholesterol-cholic acid and dianisylhexene on serum lipid concentrations appear to be additive.

The treatments used affected the ratios of total cholesterol to total lipids and of free cholesterol to total cholesterol. The addition of cholesterol and cholic acid resulted in increases in these ratios similar to those noted in Experiment 1. Dianisylhexene administration alone caused a marked reduction in the ratio of total cholesterol to total lipids, and a slight increase in the ratio of free cholesterol to total cholesterol. The decreased ratio of total cholesterol to total lipids suggests that dianisylhexene administration acts to increase the production of fractions of serum lipids other than cholesterol. The effects of a combination of cholesterol-cholic acid and dianisylhexene on the ratios of total cholesterol to total lipids and of free cholesterol to total cholesterol were similar to those observed in Experiment 1.

Level of copper in the rations fed had no appreciable influence on total and free cholesterol , or total lipid levels of the serum. The ratios of free cholesterol to total cholesterol and of total cholesterol to total lipids were not affected when copper-deficient rations were fed.

Changes in blood lipid constituents were affected by the length of treatment (Figure 2). In general, the maximum effect of treatments on blood lipid levels was attained when chicks had been fed experimental rations for four weeks. The levels of total and free cholesterol,

Table 7. Effects of treatments on serum lipid constituents and hemoglobin content of blood of chicks

Group number	Treatment	Age of chick week	Serum lipid constituents[1] TC	FC	TL	TC/TL ratio[2]	FC/TC ratio[3]	Hb content[4] g/100 ml
			mg/100 ml					
1	Control	2	173.4	22.1	753	0.23	0.13	9.78
		4	265.2	18.3	749	0.35	0.07	11.29
		6	231.5	44.6	643	0.36	0.19	11.07
		avg	223.4	28.3	715	0.31	0.13	10.71
2	As 1 + choles-terol & cholic acid	2	643.5	107.5	2097	0.31	0.17	8.66
		4	643.5	102.4	1849	0.35	0.16	11.42
		6	679.8	145.2	2186	0.31	0.21	12.32
		avg	655.6	118.4	2044	0.32	0.18	10.80
3	As 1 - copper	2	181.8	24.5	670	0.27	0.13	8.97
		4	264.6	16.8	780	0.34	0.06	11.30
		6	232.1	40.9	628	0.37	0.18	11.28
		avg	226.2	27.4	693	0.33	0.12	10.52
4	As 3 + choles-terol & cholic acid	2	623.7	84.9	1609	0.39	0.14	7.87
		4	567.5	76.4	1337	0.42	0.13	12.04
		6	645.3	97.1	1723	0.37	0.15	12.10
		avg	612.2	86.1	1556	0.39	0.14	10.67
5	As 1 + dianisy-lhexene	2	626.6	114.8	6016	0.10	0.18	8.78
		4	1332.3	281.3	12567	0.11	0.21	8.15
		6	1202.2	253.4	12860	0.08	0.21	8.89
		avg	1053.7	216.5	10481	0.10	0.20	8.61
6	As 2 + dianisy-lhexene	2	1558.8	367.7	9017	0.17	0.23	7.84
		4	1469.4	336.0	12517	0.12	0.23	9.21
		6	1797.3	408.5	13817	0.13	0.23	9.65
		avg	1608.5	370.7	11784	0.14	0.23	8.90
7	As 3 + dianisy-lhexene	2	686.1	134.4	5942	0.11	0.19	8.39
		4	1348.4	294.9	14320	0.09	0.22	8.39
		6	958.9	174.5	8575	0.07	0.18	8.77
		avg	997.8	201.3	9612	0.09	0.20	8.52
8	As 4 + dianisyl-hexene	2	1340.3	217.0	6496	0.20	0.16	7.76
		4	1646.1	429.9	12402	0.13	0.26	7.85
		6	1650.3	394.2	12602	0.13	0.24	10.03
		avg	1545.5	347.0	10500	0.15	0.22	8.55

[1]TC=total cholesterol; FC=free cholesterol; TL=total lipids.

[2]Ratio of total cholesterol to total lipids.

[3]Ratio of free cholesterol to total cholesterol.

[4]Hemoglobin content.

and of total lipids in the serum remained relatively constant between 4 and 6 weeks of age.

Figure 3 illustrates the effect of administration of dianisylhexene on total cholesterol levels of the serum at different treatment periods, i.e., at 2, 4, and 6 weeks of age, respectively. The direction of the response was the same at each period, but the magnitude of the response induced by 0.02% of dianisylhexene was significantly different, showing the lowest response to dianisylhexene at 2 weeks of age. Total cholesterol levels at 4 and 6 weeks of age were not affected to any extent by dianisylhexene treatment. Similar results were noted with respect to the influence of dianisylhexene administration on free cholesterol and total lipid fractions of the serum as shown in Figures 4 and 5, respectively.

Data pertaining to the distribution of fatty acids in serum lipids are presented in Table 8. The results obtained are similar to those observed in Experiment 1. Cholesterol and cholic acid supplementation resulted in a highly significant increase ($P < 0.01$) in the proportion of oleic acid present in serum lipids. The increase in the proportion of oleic acid was accompanied by a decrease in the proportions of palmitic, stearic and linoleic acids. Administration of dianisylhexene alone (Groups 5 and 7) caused a highly significant increase ($P < 0.01$) in oleic acid levels, a decrease ($P < 0.01$) in the proportions of stearic and linoleic acids, and an increase ($P < 0.05$) in palmitic acid. The inclusion of cholesterol-cholic acid and dianisylhexene in the rations fed resulted in a significant increase ($P < 0.05$) in palmitic acid and a further decrease ($P < 0.05$) in

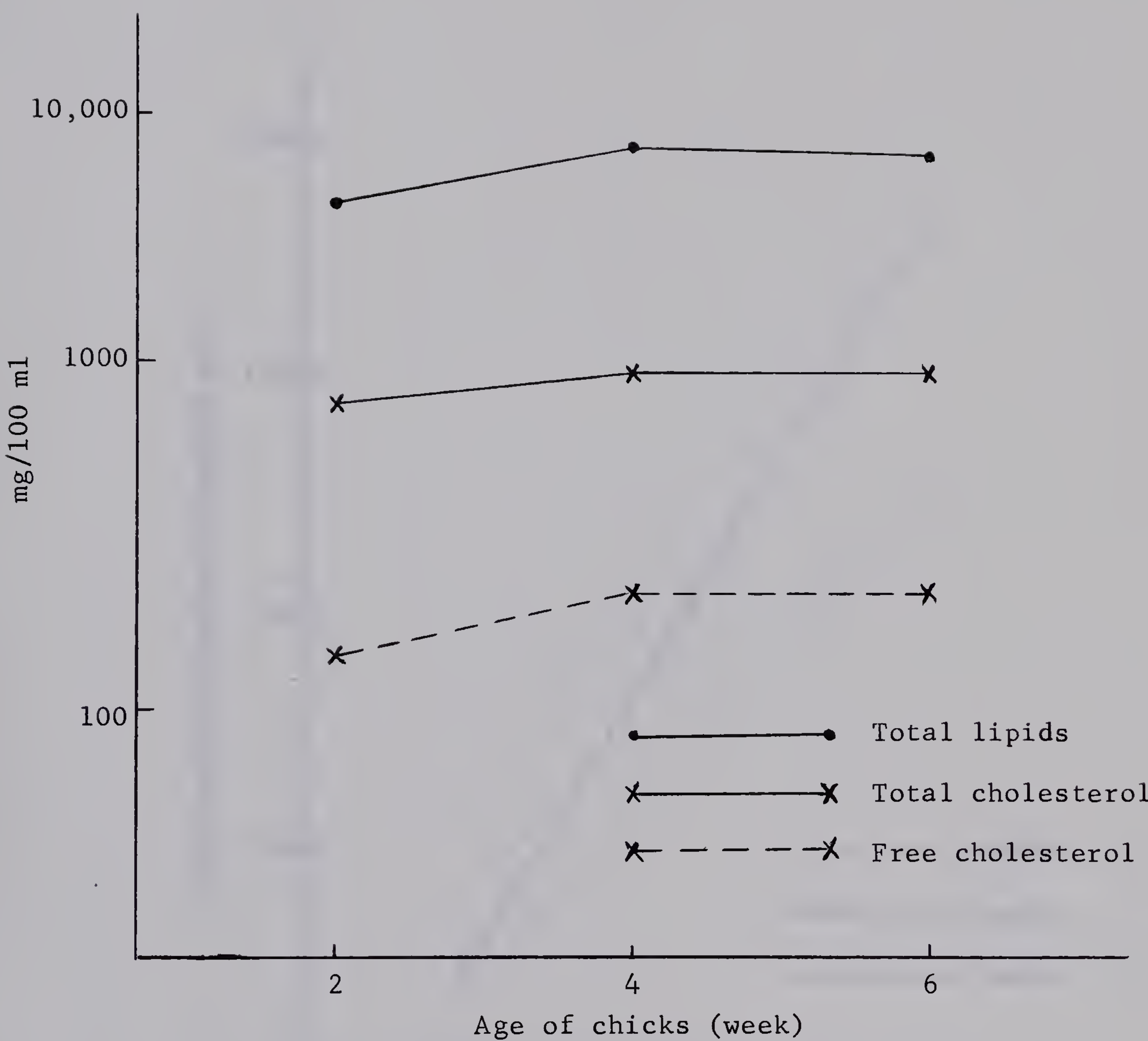

Figure 2. Interrelationship between age and serum lipid constituents.

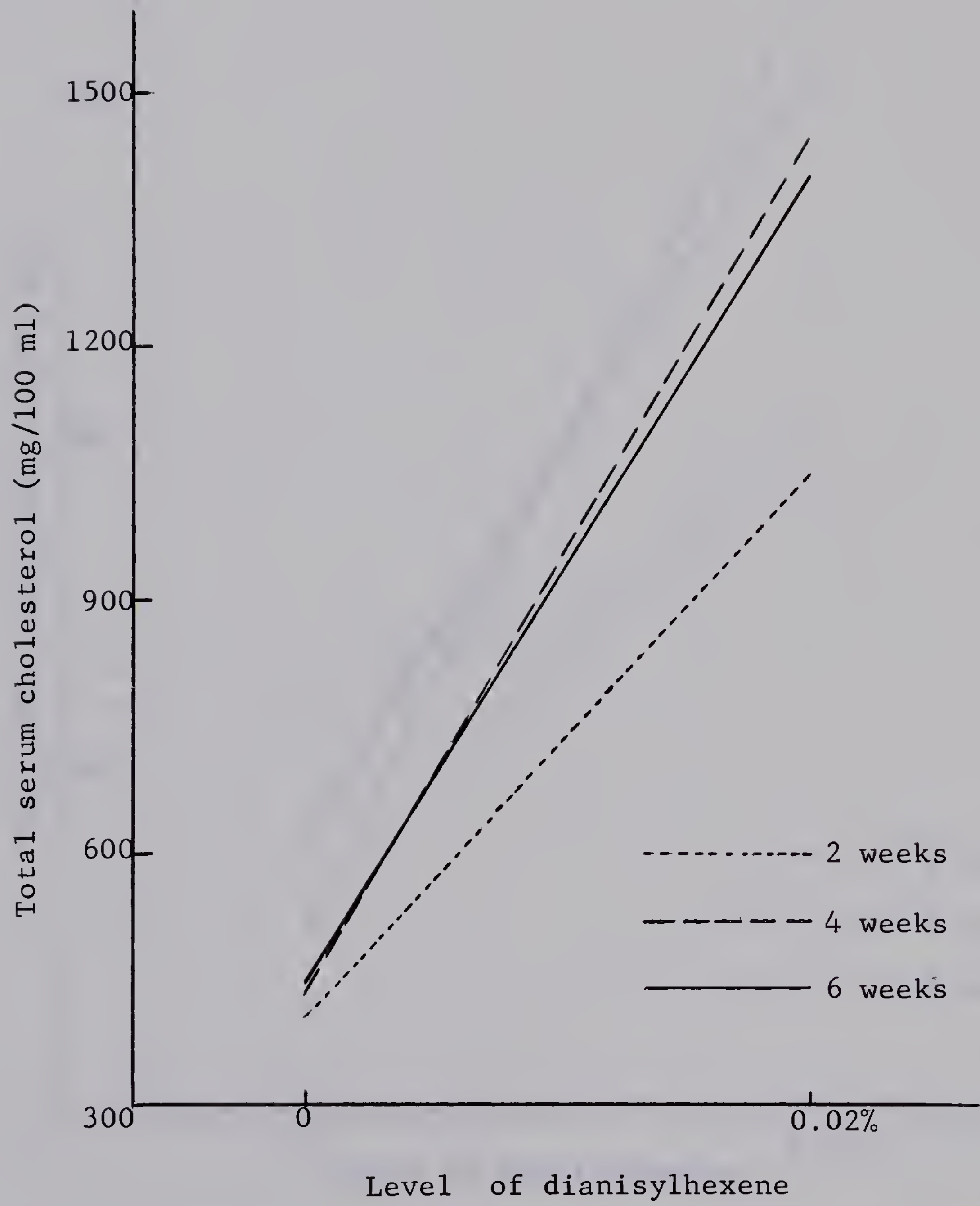

Figure 3. Interaction of age and level of dianisylhexene on total serum cholesterol levels.

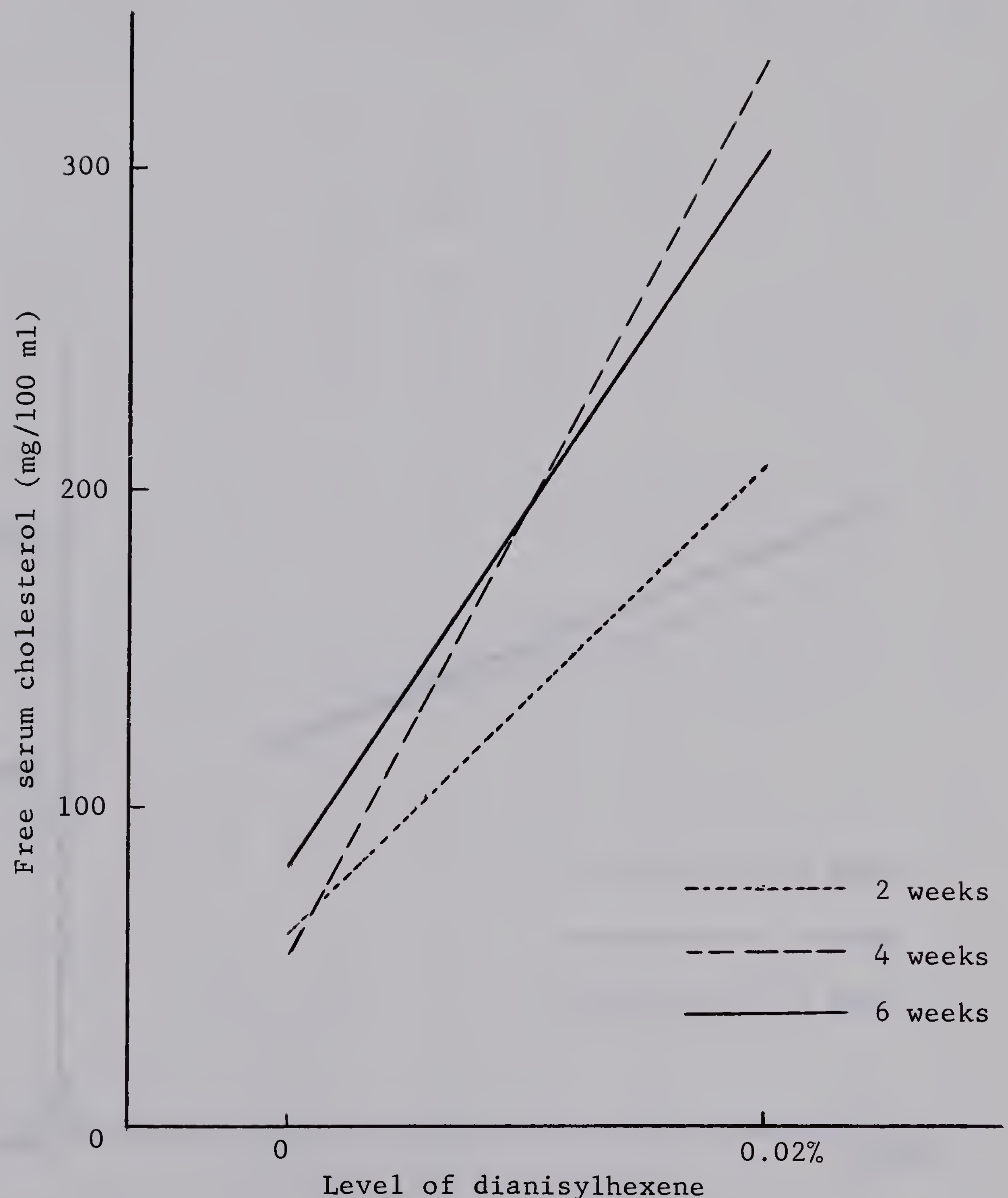

Figure 4. Interaction of age and level of dianisylhexene on free serum cholesterol levels.

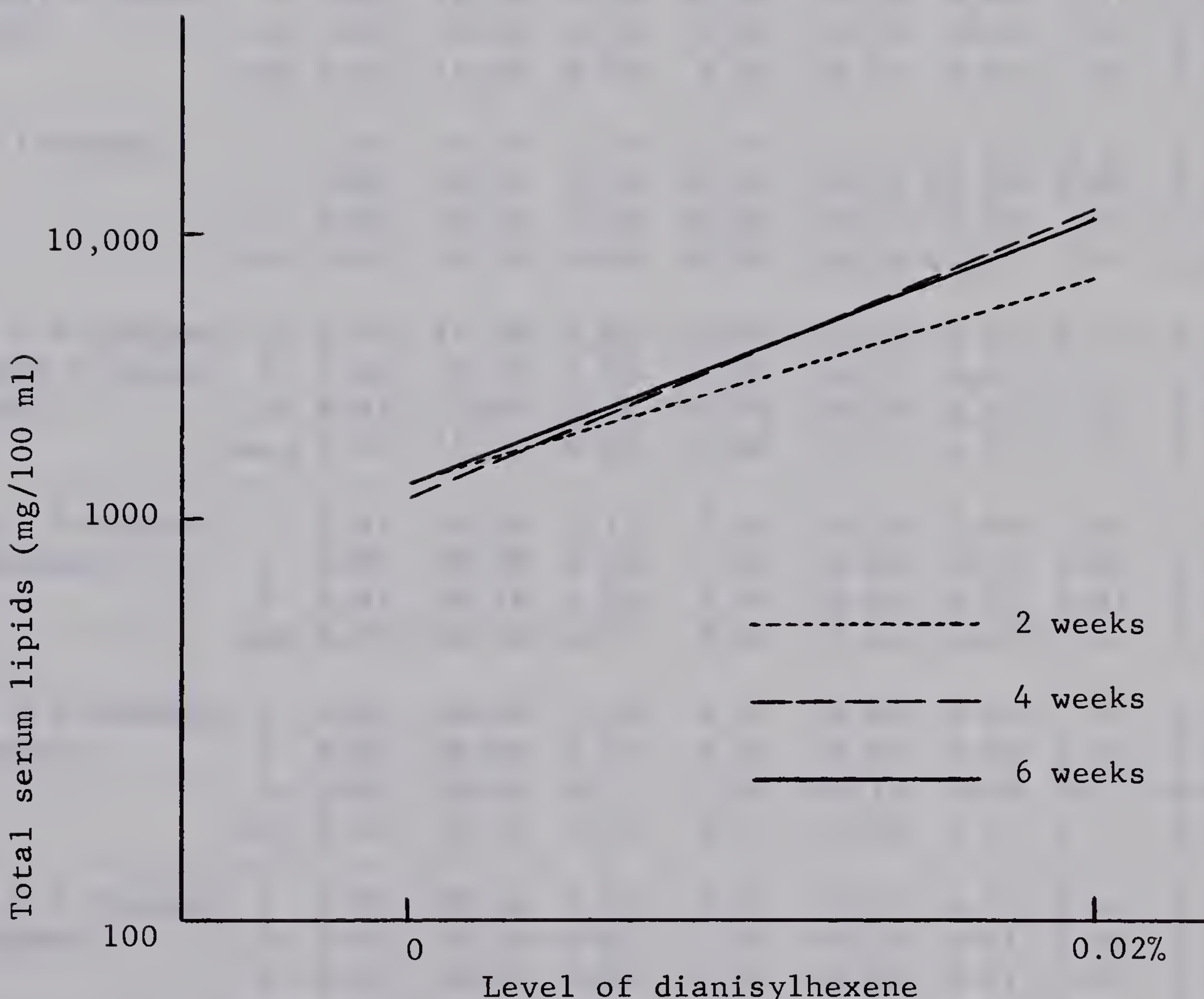

Figure 5. Interaction of age and level of dianisylhexene on total serum lipid levels.

Table 8. Effect of treatments on the distribution of fatty acids in serum lipids of chicks

Group number	Treatment	Age of chick week	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0
			Fatty acids[1] %							
1	Control	2	0.63	23.23	4.57	18.70	42.31	9.56	0.79	0.17
		4	0.57	26.51	3.79	17.98	35.98	14.04	0.87	0.24
		6	0.55	25.31	2.08	21.28	35.11	14.72	0.72	tr[2]
		avg	0.58	25.02	3.48	19.32	37.80	12.77	0.79	0.20
2	As 1 + choles-	2	0.78	19.04	6.80	11.39	51.28	8.69	1.24	0.75
	terol & cholic	4	0.61	14.70	4.29	10.09	58.48	8.96	2.16	0.69
	acid	6	0.46	12.82	3.79	7.97	64.28	8.47	1.53	0.84
		avg	0.61	15.52	4.96	9.82	58.01	8.70	1.64	0.76
3	As 1-copper	2	0.60	25.02	5.73	17.26	41.32	8.89	0.87	0.28
		4	0.89	26.69	3.04	19.70	35.33	13.50	0.39	0.54
		6	0.67	25.33	2.83	20.05	33.21	15.12	2.77	tr
		avg	0.72	25.68	3.86	19.00	36.62	12.50	1.34	0.41
4	As 3 + choles-	2	0.39	17.78	6.90	12.09	53.26	8.57	0.78	0.22
	terol & cholic	4	0.42	15.79	3.95	11.55	56.67	9.66	1.25	0.69
	acid	6	0.61	13.65	3.56	11.78	59.70	8.63	1.05	0.99
		avg	0.47	15.74	4.80	11.80	56.54	8.95	1.03	0.63
5	As 1 + dianis-	2	0.51	28.28	5.73	9.09	50.20	5.04	0.69	0.43
	ylhexene	4	0.67	29.84	5.25	7.14	51.86	4.13	0.70	0.39
		6	0.47	26.53	3.32	9.10	55.81	3.98	0.47	0.29
		avg	0.55	28.22	4.77	8.44	52.62	4.38	0.62	0.37
6	As 2 + dianisy-	2	0.52	24.40	5.38	9.73	52.08	6.24	1.01	0.64
	lhexene	4	0.52	24.68	5.42	8.56	53.82	4.90	1.60	0.47
		6	0.55	23.57	4.77	7.38	58.15	4.22	0.77	0.56
		avg	0.53	24.22	5.19	8.56	54.68	5.12	1.12	0.56
7	As 3 + dianisy-	2	0.51	29.54	5.71	9.36	49.13	4.98	0.40	0.35
	lhexene	4	0.72	28.21	5.81	7.42	52.33	4.01	0.96	0.52
		6	0.61	26.49	3.56	12.15	51.02	5.01	0.70	0.43
		avg	0.61	28.08	5.03	9.64	50.82	4.67	0.69	0.43
8	As 4 + dianisy-	2	0.58	23.80	5.28	9.42	53.44	6.65	0.56	0.50
	lhexene	4	0.56	23.61	5.32	7.63	56.03	5.16	0.89	0.77
		6	0.63	23.91	4.21	8.25	56.08	5.39	0.89	0.61
		avg	0.59	23.77	4.94	8.43	55.18	5.73	0.78	0.63

[1,2]Same as footnote in Table 4.

linoleic acid levels as compared with that of chicks receiving cholesterol-cholic acid alone (Groups 6 and 8 vs. Groups 2 and 4). This would again suggest that different mechanisms were involved in changing the composition of fatty acids in serum lipids when dianisylhexene or cholesterol-cholic acid was fed to chicks.

Examination of the data suggested that an interaction existed between dianisylhexene and cholesterol-cholic acid in their effects on the distribution of fatty acids in serum lipids. The magnitude and direction of the response of four major fatty acids, i.e., palmitic, stearic, oleic, and linoleic acids, to dianisylhexene and cholesterol-cholic acid supplementation are presented in Figures 6 to 9. It is apparent that dianisylhexene and cholesterol-cholic acid acted in different ways in altering the distribution of these fatty acids.

Copper deficiency had no appreciable influence on the distribution of fatty acids in serum lipids (Table 8). The proportion of fatty acids present in the serum remained relatively constant when similar rations were varied in copper content.

Variations in blood hemoglobin content resulting from different treatments are presented in Table 7. It was noted, as in Experiment 1, that cholesterol-cholic acid supplementation did not affect the hemoglobin concentration of blood. A deficiency of copper in the ration fed caused a slight, but non-significant, decrease in blood hemoglobin levels. It was found, however, that dianisylhexene administration did affect the hemoglobin content of blood, resulting in a significant decrease ($P < 0.05$) in all cases. The lowest hemoglobin level was noted when chicks were fed a copper-deficient ration supplemented with dianisylhexene.

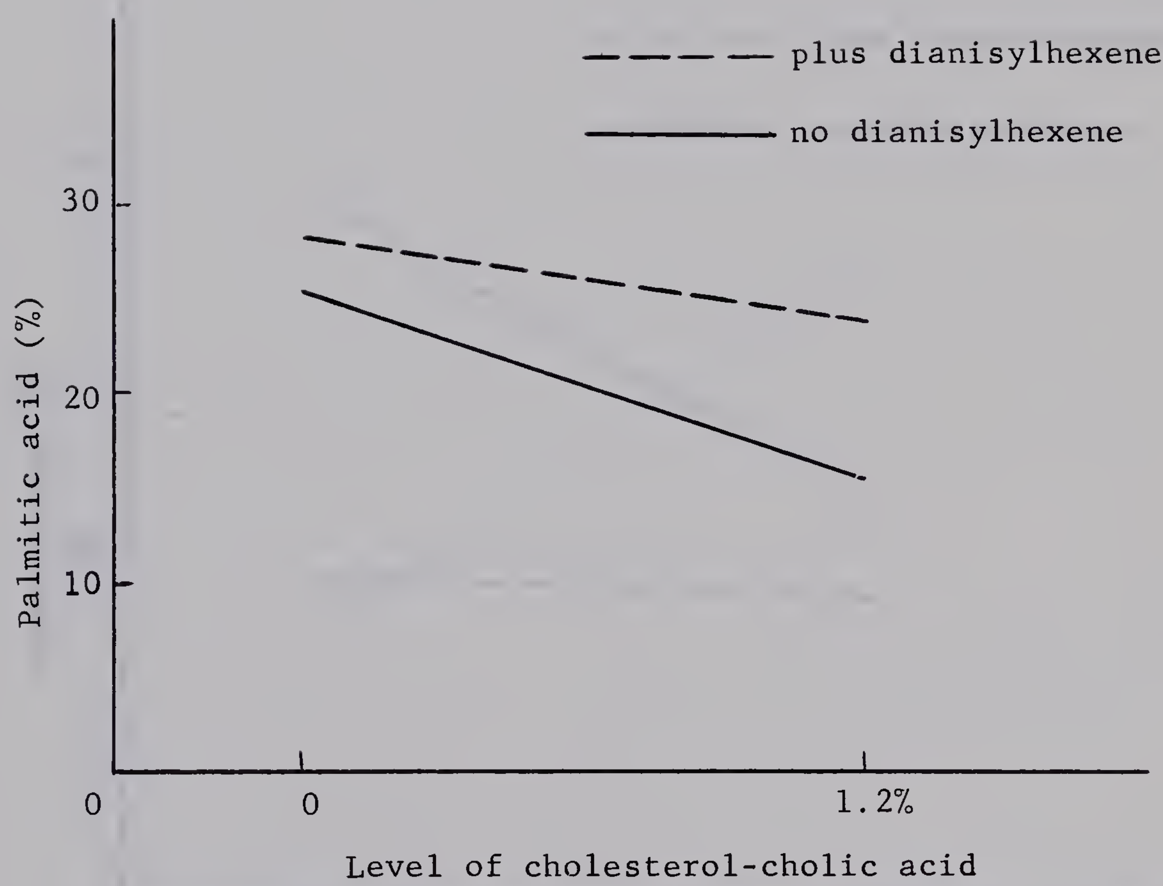

Figure 6. Interaction of dianisylhexene and cholesterol-cholic acid on palmitic acid levels.

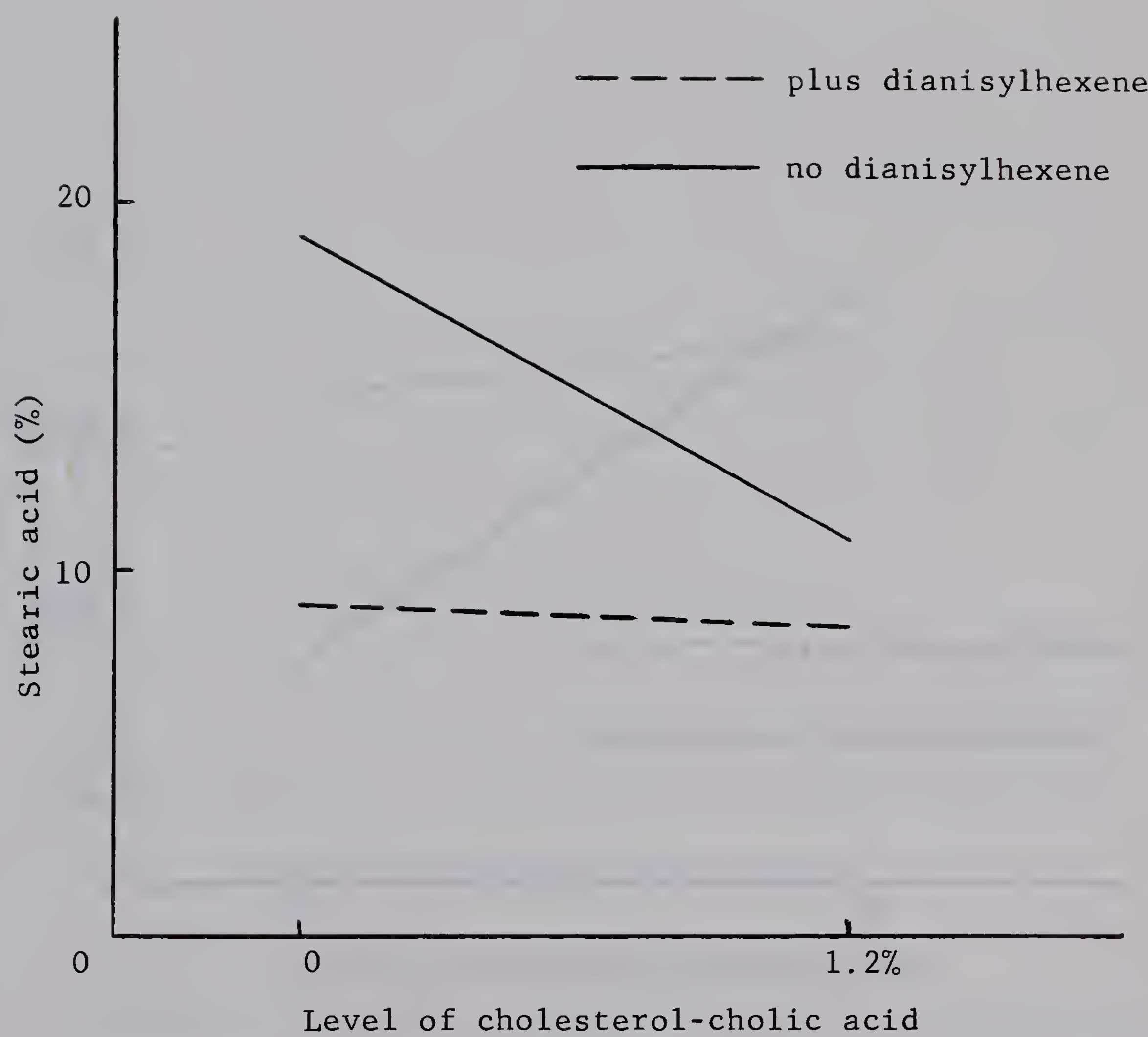

Figure 7. Interaction of dianisylhexene and cholesterol-cholic acid on stearic acid levels.

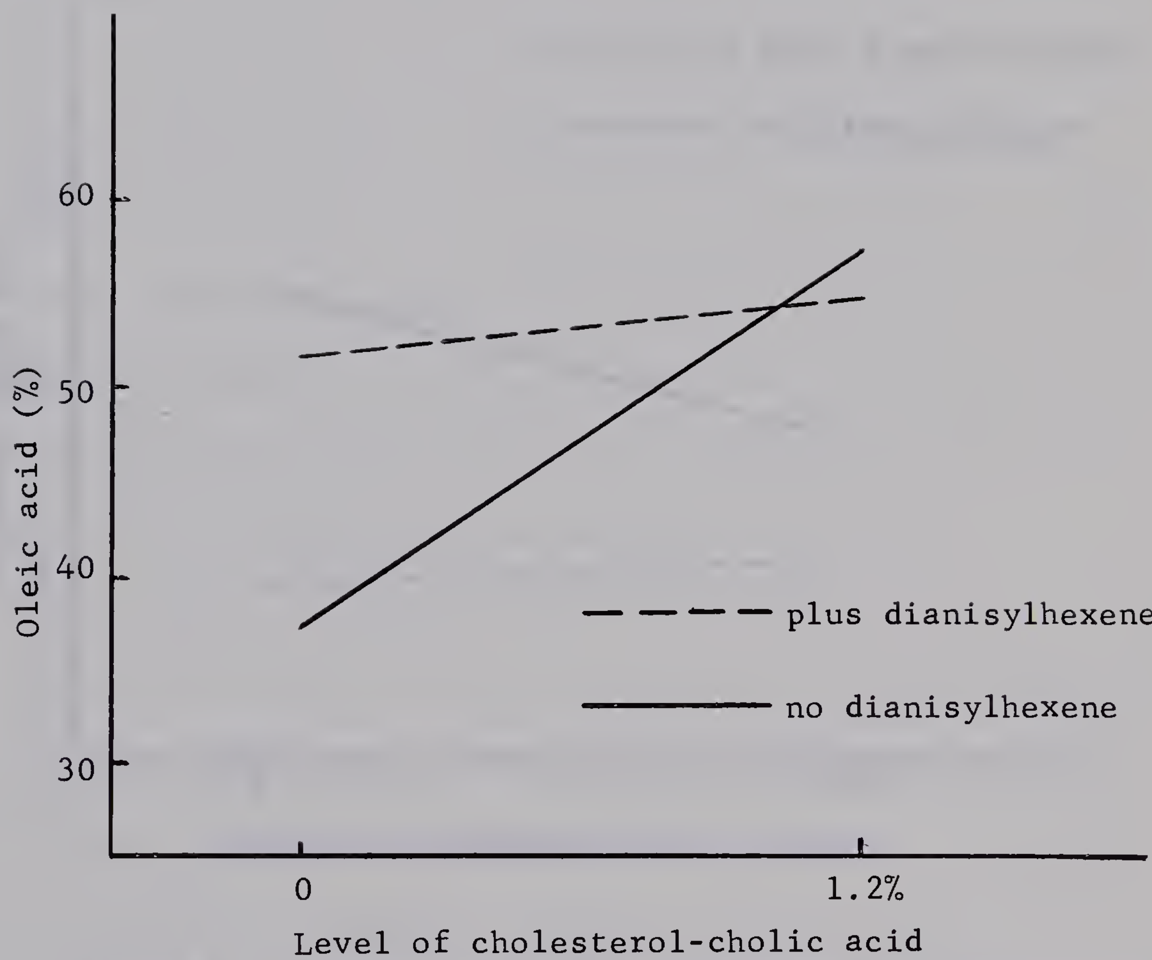

Figure 8. Interaction of dianisylhexene and cholesterol-cholic acid on oleic acid levels.

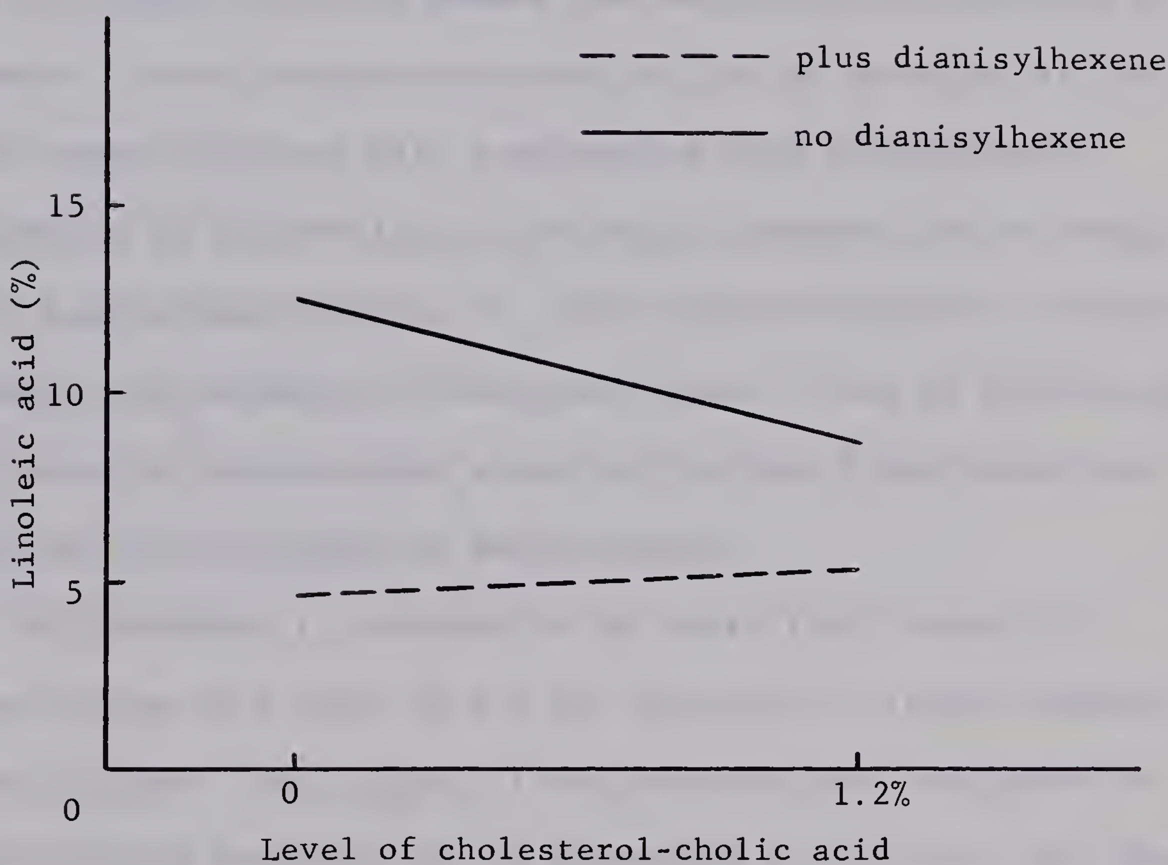

Figure 9. Interaction of dianisylhexene and cholesterol-cholic acid on linoleic acid levels.

The effects of treatments on body weight, mortality and incidence of aortic rupture are shown in Table 9. Dianisylhexene administration resulted in a decrease in rate of growth, but cholesterol-cholic acid feeding or level of copper in the ration had little effect on weight gains. Level of mortality varied between treatments with the highest rate of mortality occurring in the groups fed copper-deficient rations supplemented with dianisylhexene. Post-mortem examination of the chicks that died showed that aortic rupture occurred in two treatments. Aortic rupture accounted for 11% of mortality in the group fed a copper-deficient diet supplemented with dianisylhexene (Group 7) and for 4% of mortality in the copper-adequate ration supplemented with dianisylhexene (Group 5). Since copper-deficiency has been shown to affect the integrity of vascular system, it may be postulated that a low level of copper intake along with extreme hyperlipemia was responsible for the occurrence of aortic rupture.

As in Experiment 1, sections of the aorta from 6 week-old chicks were stained with Sudan IV for the detection of lipids deposited in the aortic intima. Once again, it was observed that the extent of lipid deposition was dependent on the treatments used (Figure 10). The extent of lipid deposition, reflected by the density of Sudan IV staining, was increased when cholesterol-cholic acid or dianisylhexene, alone or in combination, was administered . Dietary copper levels apparently had no effect on the degree of lipid deposition in the aorta. This differed from the results of O'Dell et al. (1966) who reported that the aortas from chicks fed copper-deficient rations appeared to contain higher percentage of lipids than did the controls. No explanation for the difference in results can be offered.

Table 9. Effects of treatments on body weight, mortality and incidence of aortic rupture.

Group number	Treatment	Body weight 6 weeks	Mortality	Incidence of aortic rupture
		g	%	%
1	Control	539	4	0
2	As 1 + cholesterol & cholic acid	459	11	0
3	As 1 - copper	515	11	0
4	As 3 + cholesterol & cholic acid	524	15	0
5	As 1 + dianisylhexene	355	15	4
6	As 2 + dianisylhexene	368	4	0
7	As 3 + dianisylhexene	386	26	11
8	As 4 + dianisylhexene	341	26	0

Figure 10. The effect of treatments on lipidosis of the aorta of 6 week-old chicks (Experiment 2).

Summary

1) Administration of cholesterol and cholic acid or dianisylhexene to chicks resulted in an increase in levels of total and free cholesterol, and of total lipids in the serum; dianisylhexene was more effective than cholesterol-cholic acid. A combination of dianisylhexene and cholesterol-cholic acid resulted in the highest concentrations of total and free cholesterol, and of total lipids in the serum.

2) Addition of cholesterol-cholic acid or dianisylhexene to the ration of chicks caused an increase in the ratio of free cholesterol to total cholesterol in the serum. A combination of cholesterol-cholic acid and dianisylhexene resulted in a further increase in the ratio. The ratio of total cholesterol to total lipids was elevated slightly by feeding of cholesterol and cholic acid, but was reduced markedly when dianisylhexene was administered.

3) Feeding of cholesterol and cholic acid resulted in a highly significant increase in the proportion of oleic acid and a decrease in linoleic, stearic and palmitic acids in serum lipids. Dianisylhexene administration had a somewhat similar effect except that the magnitude of change in the proportions of fatty acids differed and an increase in the proportion of palmitic acid present occurred.

4) A deficiency of copper in the ration fed had no appreciable effect on serum lipid levels or distribution of fatty acids in serum lipids.

5) Inclusion of dianisylhexene in the ration fed resulted in a significant decrease in the hemoglobin levels of blood. A deficiency of copper caused a slight, but non-significant, decrease in the hemoglobin levels.

6) Level of mortality was high in chicks fed a copper-deficient diet supplemented with dianisylhexene. Aortic rupture was responsible for 11% of mortality in the group.

7) The addition of dianisylhexene to the ration fed resulted in a depression in rate of growth of chicks.

GENERAL DISCUSSION

Hyperlipemia and hypercholesterolemia were induced in chicks by feeding rations containing cholesterol (1%) and cholic acid (0.2%) or dianisylhexene (0.02%). The severity of hyperlipemia and hypercholesterolemia noted was increased when dianisylhexene was administered, and was most severe when a combination of cholesterol-cholic acid and dianisylhexene was used. These results are in agreement with observations reported previously (see Review of Literature).

The ratio of free cholesterol to total cholesterol remained relatively low in these experiments. This would indicate that most of the cholesterol in the serum was in the esterified form, presumably esterified during absorption or in the tissues. The low percentage of free cholesterol noted may have been influenced by the high level of oleic acid contributed by the fat added to the rations used. Karmen, Whyte, and Goodman (1963) noted that during absorption from the intestinal tract, cholesterol was esterified, preferentially with oleic acid, in the intestinal mucosa. Vahouny and Treadwell (1959) observed that the 18-carbon fatty acids, stearic, oleic and linoleic, stimulated cholesterol absorption, with oleic acid exhibiting the most marked effect. There was no evidence in the present experiment that the ratio of free cholesterol to total cholesterol was correlated with levels of oleic acid in serum lipids.

The inclusion of cholesterol and cholic acid in the rations fed resulted in an increase in the ratio of total cholesterol to total lipids, but administration of dianisylhexene resulted in a marked decrease in the ratio. This implies that these compounds caused

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

hyperlipemia to develop in different ways. It has been reported (Stamler and Katz, 1950) that feeding of cholesterol predominantly promoted an increase in the blood cholesterol fraction rather than other lipid fractions of blood, and that the administration of estrogen (Lorenz et al., 1938; Entenman et al., 1940;Lindsay et al., 1946) resulted in a hyperlipemia characterized by a marked increase in the neutral fat fraction of plasma lipids. Changes that were observed in the ratios of total cholesterol to total lipids in the present experiment may be readily reconciled on the basis of the above reports.

The mechanisms involved in the development of hyperlipemia and hypercholesterolemia as a result of administration of estrogenic hormones have not been elucidated. It is possible that estrogen functions by improving absorption of lipids, by promotion of endogenous synthesis of lipids from other sources, such as carbohydrate and protein, or by changing the rate at which tissue lipids are metabolized. Regardless of the mechanisms involved the administration of dianisylhexene resulted in the occurrence of very high blood lipid levels.

The distribution of fatty acids in the serum lipids of chicks was influenced by the treatments used in the experiment. Inclusion of cholesterol-cholic acid or dianisylhexene in the rations fed resulted in a significant increase in the proportion of oleic acid and a decrease in stearic acid. Although both treatments caused similar changes in the distribution of the two fatty acids, the extent of the change differed slightly between treatments. Levels of linoleic and palmitic acids were also affected, but in different ways. Supplementing the ration with cholesterol and cholic acid resulted in a decrease in the proportions of linoleic and palmitic acids present in the serum, whereas

administration of dianisylhexene resulted in a decrease in the proportion of linoleic acid present and an increase in the proportion of palmitic acid. The decrease in the proportion of linoleic acid was greater when dianisylhexene was used than when cholesterol-cholic acid was added to the ration. Thus, it would appear that different mechanisms were involved in altering the distribution of fatty acids in serum lipids.

The combined effect of cholesterol-cholic acid and dianisylhexene in changing the distribution of fatty acids was not additive, but rather represented an average of the response to each treatment alone. This agrees with the observations of Chung et al. (1966) who noted a similar change in fatty acid distribution as a result of the administration of cholesterol and diethylstilbestrol.

A small percentage of mortality attributable to aortic rupture was noted in the second experiment. This occurred in two groups, one receiving dianisylhexene and the others receiving dianisylhexene in a ration deficient in copper content. This suggests that both dianisyl-hexene and a deficiency in copper may contribute to derangement of the vascular system, and hence, to the occurrence of aortic rupture.

GENERAL SUMMARY

Two experiments were conducted to study the effects of dietary and hormonal factors on blood lipid levels, fatty acid composition of serum lipids, blood hemoglobin content, lipidosis of the aorta, and occurrence of aortic rupture in chicks. A summary of the results obtained is presented below:

1) Cholesterol and cholic acid supplementation of rations for chicks resulted in a significant increase ($P < 0.05$) in total and free cholesterol and a pronounced (statistically non-significant) increase in total lipid levels of the serum. A highly significant increase ($P < 0.01$) in the proportion of oleic acid (18:1), which was accompanied by decreases in the proportions of linoleic (18:2), stearic (18:0) and palmitic (16:0) acids, was noted in chicks receiving cholesterol and cholic acid.

2) The addition of dianisylhexene (0.02%), an estrogenic hormone, to the ration fed caused severe hyperlipemia and hypercholesterolemia characterized by extremely high levels of total and free cholesterol, and of total lipids in the serum. Changes in the distribution of oleic, stearic and linoleic acids present in serum lipids were similar to changes caused by feeding of cholesterol and cholic acid, but the magnitude of change in the proportions of these fatty acids differed. In addition, dianisylhexene resulted in an increase in the proportion of palmitic acid present, while cholesterol and cholic acid feeding resulted in a decrease.

3) The highest levels of total and free cholesterol and total lipids in the serum were obtained when chicks were fed cholesterol-

cholic acid containing rations supplemented with dianisylhexene.

4) The ratio of free cholesterol to total cholesterol was elevated when chicks were fed rations supplemented with cholesterol and cholic acid or dianisylhexene. Administration of dianisylhexene resulted in a marked decrease in the ratio of total cholesterol to total lipids in the serum, whereas feeding of cholesterol and cholic acid resulted in a slight increase.

5) The inclusion of cholesterol-cholic acid, or dianisylhexene in the rations fed resulted in lipidosis of the aorta of chicks. The most severe lipidosis was noted when a combination of cholesterol-cholic acid and dianisylhexene was fed. Levels of magnesium and copper in the diet apparently had no effect on the degree of lipidosis that occurred.

6) Suboptimal level of dietary magnesium and a deficiency of copper in the rations fed had no appreciable effect on serum lipid levels or on the distribution of fatty acids in serum lipids of chicks.

7) The addition of dianisylhexene to the ration fed resulted in a depression in the hemoglobin content of whole blood.

8) The administration of dianisylhexene caused a depression in rate of growth of chicks.

9) Aortic rupture occurred in two groups of chicks. The highest level of mortality attributable to aortic rupture occurred in the group receiving dianisylhexene in a ration deficient in copper content. One case of aortic rupture was noted in a group of chicks receiving dianisylhexene alone.

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